THE SIGNIFICANCE AND QUANTITATIVE MEASUREMENT OF THE NITROGENOUS METABOLISM OF BACTERIA

STUDIES IN BACTERIAL METABOLISM. LVII

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One of the significant indications of a transition in the biologics from the condition of an art to the status of a science is the development within them of suitable quantitative methods. This follows because qualitative observations, frequently more striking than quantitative measurements, require the application of methods of precision for their elucidation. Thus, the phenomena of growth and repair in health and disease have been explored through the rise of the science of nutrition and metabolism. The development of the science of pharmacology followed logically the perfection of methods for recording accurately the physiologic effects of drugs and chemicals. The physiologic assay of the potency of bacterial toxins has progressed along lines previously employed in pharmacology. Similarly, the rise of the youngest of the biologic sciences, immunology, has been dependent on the acquisition of procedures sufficiently accurate to estimate the nature and extent of those complex relationships which exist between microbe and host, in infection and in resistance to infection. Furthermore, the determination of the potency of lysins, agglutinins and precipitins has assumed practical significance in medicine since their numerical relations to infection and resistance have been established.

The application of quantitative chemical methods to the study of bacterial metabolism is in its infancy. This might confidently be expected because qualitative observations involving phenomena of bacterial metabolism are few in number, inconspicuous in their relations to medicine, in whose domain they have largely been associated, and difficult of recognition. Nevertheless, some results have been recorded which not only shed light on the mode of action of bacteria, but also indicate lines of approach to underlying and fundamental principles of cellular behavior in general.

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Among the available indications of the possible results that may flow from the study of bacterial chemistry, the observations of Theobald Smith [1] that glucose in cultures of the diphtheria bacillus prevents the formation of diphtheria toxin, may be cited. This phenomenon is an example of a great group of related reactions which seem to depend on the physiologic dictum that "carbohydrate spares protein from utilization for energy." [2] Stated differently, the specificity of action of a majority of pathogenic bacteria depends on their utilization of protein for energy.[3] Simonds'[4] study of the relation of the composition of mediums to the immunologic reactions of bacteria developed therein is an important contribution to the same field. The effects of cultural sources of energy on the development of soluble enzymes [5] is still another indication of the relationship that exists between the nature of the nutrition of bacteria and the character of their activities. The ever-increasing developments along chemical lines in those industries in which bacteria play a part is a suggestion of the capitalization of utilitarian bacterial transformations in processes of economic importance. Even in the domain of therapeutics there is evidence that a knowledge of bacterial metabolism may prove to be the starting point for dietary procedures of importance in the treatment of bacillary dysentery, [3, 6] typhoid fever,[7] and other excrementitious diseases.

THE NATURE OF BACTERIAL METABOLISM

The life history of a bacterium, like that of any living entity, may be divided into two distinct phases, the structural phase and the energy phase. The structural phase comprises those phenomena which result in the transformation of suitable nutrient substances, together with water and salts, into the mature bacterial cell. The structural phase is complete, aside from losses incidental to the formation of enzymes and other nitrogenous substances necessary for the maintenance of the organism, when the phenomenon of fission from the mother cell is complete. The amount of substance required to build a bacterial cell is little indeed; an average sized typhoid bacillus weighs about 0.000,000,002 of a milligram, its volume being very nearly one two-thousand-millionth of a cubic millimeter.[8] In other words, two million

[1] Jour. Exper. Med., 1899, 4, p. 373.
[2] Kendall: Chem. and Metall. Jour., 1921, 24, p. 56.
[3] Kendall: Am. Jour. Med. Sc., 1918, 156, p. 157.
[4] Jour. Infect. Dis., 1915, 17, p. 500.
[5] Auerbach: Arch. f. Hyg., 1897, 31, p. 311. Kendall and Walker: Jour. Infect. Dis., 1915, 17, p. 442.
[6] Kendall: Boston Med. & Surg. Jour., 1911, 154, p. 288.
[7] Torrey: Jour. Med. Res., 1919, 39, p. 415.
[8] Kendall: Med. Rec., 1913, 84, p. 151.

million typhoid bacilli would be required to balance a gram weight. About 87% of this is water. The amount of organic substance is comparatively small.

The energy requirements of bacterial cells are as disproportionately extensive as the structural requirements are inconspicuous. The surface area of the typhoid bacillus mentioned in the foregoing, whose weight is one two-thousand-millionth of a milligram, is one hundred-thousandth of a square millimeter. Expressed numerically, the surface area of the microbe to its weight is as 0.000,01 is to 0.000,000,002.[9] As the energy requirements of living things vary with the surface area rather than with the volume, the well-known ability of bacteria to effect transformations of nutritive material far in excess of the amount their extremely minute size would apparently permit, receives some explanation. The rapid development of bacteria in favorable mediums, in which successive generations may appear at intervals as frequent as every 15 or 20 minutes in the early hours of growth in a fresh environment furnishes the factor of enormous numbers of microbes which is necessary to complete the background for those rapid chemical transformations, which bacteria are capable of.

Nitrogen is the corner stone of the structural requirement of living things, and bacteria are of course no exception. Nitrogen in available form is absolutely essential for the growth of bacteria. The significant element in the energy phase of bacterial metabolism, however, is carbon; nitrogen not only is not a source of energy for most bacteria, but it also is useless for this purpose. This is equally true, so far as available information indicates, in the animal kingdom. The well-known utilization of available carbohydrates (which possess the requisite stereo-configuration to fit the bacterial cytoplasm) for energy in mediums containing both protein derivatives and sugars is illustrative of this fact. There is no nitrogenous residuum in carbohydrate, and the partially oxidized carbon of the sugar molecule possessing the requisite stereo-configuration appears to be a peculiarly readily utilized source of energy. If, however, the carbohydrate is withdrawn from the medium, leaving the protein derivatives alone for both the structural (nitrogenous) and energy (carbonaceous) requirements, the nitrogen of the amino acids and polypeptids is eliminated quantitatively as ammonia (deamination prior to the utilization of the non-nitrogenous residium for energy). In this instance, the amount of ammonia

[9] For purposes of comparison, a man 200 centimeters tall, weighing 100 kg. has a surface area of almost exactly 2.36 square meters.

accumulating as a result of deamination is a very exact measure of the intracellular utilization of amino acids or their complexes for bacterial energy.[10]

It is a well attested fact that the amount of urea excreted from the animal body increases with the increase of protein in the diet above that minimum necessary to establish nitrogen equilibrium; therefore, a purely protein diet is associated with much urea excretion. The substance antecedent to urea is of course ammonia, which results from the deamination of amino acids or their complexes. In this sense, urea excreted in the urine of mammals and ammonia excreted in cultures of bacteria have an almost parallel significance.

Unfortunately, no methods are available for directly measuring the amount of carbon utilized for energy either in animals or bacterial cultures. It is possible, however, to follow the change in the nitrogenous constituents of bacterial cultures utilized for structure, and for structural repair, as they are liberated through recessive and autolytic changes, and as they are liberated from proteins or protein derivatives incidental to their intracellular utilization for energy (deamination).

Folin has devised two important methods for the measurement of nitrogenous substances, one for the determination of ammonia by the air current method, the other for nonprotein nitrogen, which in connection with the Sörensen formol titration method or the Van Slyke amino nitrogen method, and the Kjeldahl method for total nitrogen, make it possible to divide the nitrogenous constituents of culture mediums into several fractions, as follows: Knowing the total nitrogen and the total nonprotein nitrogen, it follows that their difference may be designated appropriately "protein nitrogen." Also, the nonprotein nitrogen may be fractionated into ammonia nitrogen, amino nitrogen (corrected of course for ammonia) and residual nonprotein nitrogen. In ordinary cultural mediums the nonprotein residual nitrogen may consist—in addition to polypeptids, the most important constituents—of creatin or creatinin, urea, uric acid (the latter two in traces at most), purin bases and possibly also pyrimidin bases. Mediums containing meat extract as a basis are richer in creatinin and purin bases than mediums compounded from "meat juice." In any event, no great error is introduced if the term "polypeptid nitrogen" be substituted for residual nonprotein nitrogen, and the term will be so used in this and subsequent communications.

[10] Kendall and Walker: Jour. Infect. Dis., 1915, 17, p. 442.

As an example of the fractionation of nitrogen on this basis, the composition of three samples of gelatin are appended:

TABLE 1

COMPOSITION OF THREE SAMPLES OF GELATIN

Milligrams per 100 c c Sample	Sample A	Sample B	Sample C
Total nitrogen	0.672	1.001	1.064
Protein nitrogen	0.542	0.721	0.762
Nonprotein nitrogen	0.130	0.280	0.302
Polypeptid nitrogen	0.102	0.209	0.213
Amino nitrogen	0.020	0.039	0.032
Ammonia nitrogen	0.008	0.032	0.057

A, 5 % gelatin in water, no peptone or meat extractives.
B, plain nutrient gelatin (5% gelatin, 1% peptone, 0.3% meat extract).
C, another lot of plain, nutrient gelatin prepared as under B.

It will be seen that the combined amino nitrogen and ammonia nitrogen comprise less than 10% of the total nitrogen.

It is highly important, as will be indicated in succeeding studies, to determine changes in nitrogen distribution during experiments in bacterial metabolism. The intermediary metabolism, especially experiments in bacterial metabolism between the protein and polypeptid nitrogen fractions, furnishes much information concerning the nature of bacterial attack on the nitrogenous constituents under various conditions.

It should be mentioned in passing that the creatinin fraction of the "polypeptid" nitrogen section may be determined by the colorimetric method of Folin.[11] Some bacteria [12] act on creatinin. Also, the purin and pyrimidin bases may be estimated separately, if desired.

METHODS

Total Nitrogen.—Total nitrogen is determined either by the Folin-Farmer micro method,[13] making duplicate determinations, or by the Gunning modification of the Kjeldahl procedure. If nonprotein nitrogen determinations are to be made on the same sample, the latter is somewhat less time-consuming. The procedure is as follows:

To 10 c c of the sample to be analyzed, discharged into a 300 c c pyrex Kjeldahl flask, add 5 gm. of C. P. potassium sulphate, 5 drops of a 10% copper sulphate solution, and 15 c c of nitrogen-free sulphuric acid. Digestion is practiced until the residual solution is clear, but bluish-green in color. The digestion mixture is cooled to the point of commencing viscosity; then about

[11] Jour. Biol. Chem., 1914, 17, p. 463.
[12] Popoff: Centralbl. f. Bakteriol., 1890, 7, p. 585. Antonoff: Ibid., I, O., 1907, 43, 209. Burri and Andrejew: Ibid., 1910, 56, p. 217.
[13] Jour. Biol. Chem., 1912, 11, p. 493.

50 c c of distilled water are added, and the contents poured carefully into a 100 c c graduated pyrex flask. After cooling, enough water is added to the flask in small amounts to bring the entire volume of washings to exactly 100 c c. Duplicate portions of 2 c c each are placed in 8x1 inch pyrex test tubes, enough NaOH added to make the solution strongly alkaline,[14] and the free ammonia is blown out into 250 c c flasks, each containing exactly 10 c c of N/50 HCl., a drop of alizarin, and enough water to make a total volume of about 75 c c, using the apparatus and procedure described in the following, under the Folin air current method for ammonia. It will be seen that the only departure from the classical process is the dilution of the digestion mixture, and the subsequent "blowing out" of the ammonia from an aliquot part, in duplicate or in triplicate, in place of the ordinary steam distillation of the entire amount.

As the duplicates agree, with ordinary care, within 0.10 c c of N/50 alkali, it is clear that the advantage of this method of "blowing out" the ammonia in duplicate aliquot parts is to afford a check on the entire process.

It is customary in this laboratory to run duplicate samples, both in the digestion itself, and on the subsequent determination of ammonia, from each digestate. This procedure controls both the uniformity of digestion and the accuracy of the determination of the ammonia. The satisfaction which flows from the checking of duplicate determinations on duplicate samples far outweighs the slight additional expenditure of time required to effect this procedure.

There is no criterion for the computation of the total nitrogen content of organic compounds such as cultural mediums. It is quite probable that certain combinations of carbon and nitrogen may exist, or be formed during the digestion process, which fail to be broken up by the combination of sulphuric acid and temperature of digestion. These cyanogen compounds, if they exist, are in all probability small in amount, and as the total nitrogen determination holds only for one particular lot of medium, and is repeated for each succeeding lot made from essentially the same ingredients put together in substantially the same way, the error is relatively constant, and therefore relatively insignificant. Of greater consequence is the concentration of nitrogenous substances in cultural mediums due to the loss of water by evaporation, as incubation proceeds. The practice of weighing all cultural mediums in their containers at the start of an experiment, and of restoring the loss due to evaporation before making analyses, has much to commend it.

Nonprotein Nitrogen.—The method devised by Folin and Wu [15] for the determination of "nonprotein nitrogen" in the blood has been found to be very satisfactory for the estimation of the nonprotein

[14] A drop of alizarin is added as an indicator.

[15] Jour. Biol. Chem., 1919, 38, p. 81.

nitrogen of bacterial cultures. The method in essence consists of two stages: first, the precipitation of molecules of nitrogenous substance of a rather definite but of course unknown size from solution; and, second, the determination of the nitrogen in the filtrate (which contains "nonprotein nitrogen") after the larger sized molecules are removed by filtration.

The academic question of the nature or size of nitrogenous molecules in solution in cultural mediums which are precipitated by the Folin reagent is left to others for elucidation. The remarkable fact that a quantitative separation of the constituents of a gelatin medium, containing approximately 5% of gelatin, 1% of peptone, and 0.3% of meat extractives may be made in duplicate, with astonishingly exact results, is shown in table 2, in which are shown the results when seven samples of gelatin, containing the same nitrogenous ingredients but with ascending amounts of glucose were digested in duplicate, and the nitrogen of each digestion determined in duplicate, according to the procedure outlined.

TABLE 2

GELATIN

Sample	Plain	0.1 Glucose	0.2 Glucose	0.3 Glucose	0.4 Glucose	0.5 Glucose	0.75 Glucose	
Titration..... a	8.40	8.45	8.55	8.50	8.50	8.55	8.50	Digestion A
	8.45	8.45	8.40	8.50	8.45	8.40	8.40	
Titration..... b	8.40	8.40	8.40	8.40	8.50	8.50	8.40	Digestion B
	8.50	8.40	8.50	8.45	8.50	8.50	8.55	
Average milligrams nonprotein nitrogen per 100 c c of medium	8.45	8.40+	8.45	8.45+	8.50	8.50	8.45	
	0.347	0.358	0.347	0.347	0.347	0.336	0.347	

The figures represent the number of cubic centimeters of N/50 alkali required to neutralize 10 c c of N/50 HCl., into which there has been blown the ammonia resulting from the digestion of the various samples (by the Folin air current method), in accordance with the analytic procedure soon to be described.

The analysis of many samples of gelatin has given results which are consistent, and there appears to be no doubt that the Folin nonprotein method, if followed with reasonable care, will furnish results which have a definite value in the quantitative study of the nitrogenous

metabolism of bacterial cultures. Of course the "nonprotein nitrogen" fraction will decrease somewhat and the "protein nitrogen" fraction will increase somewhat as bacterial multiplication results in the formation of bacterial protein. Also, the bacterial formation of mucin or mucin-like substances may add to the "protein nitrogen" fraction.

The filtration or centrifugalization of such cultures will separate the "bacterial protein nitrogen" from the soluble "protein nitrogen," should such a procedure be desirable. It is also clear that autolytic or recessive processes in old bacterial cultures will probably result in a decrease in protein nitrogen during the later stages of incubation. Such recessive changes may be expected after seven or eight days' continual development at body temperature.

Procedure: Ten c c of culture are diluted with exactly 70 c c of distilled water, and thoroughly mixed. Exactly 10 c c of a 10% aqueous solution of sodium tungstate are added with constant agitation, and then exactly 10 c c of ⅔ normal sulphuric acid. The resulting solution (100 c c) should be faintly acid to congo red paper. Continuous agitation is practiced for several minutes. The turbid solution containing a tenacious precipitate is filtered through a moderately coarse filter paper. The filtrate is usually clear at first, but soon becomes milky, due apparently to the formation of an oxidization product of tungsten. Such a filtrate containing glucose frequently becomes blue on standing in the sunlight.

Digestion:[16] 25 c c duplicate samples of the filtrate are placed in 300 c c pyrex digestion flasks. To each is added about 15 c c nitrogen-free sulphuric acid, about 5 gm. potassium sulphate, and 5 drops of a 10% solution of copper sulphate. Digestion is carried out in precisely the same manner as that for the determination of total nitrogen.

When digestion is complete and the contents of the flasks have cooled somewhat, the digestion mixture is made up to a volume of exactly 100 c c (at room temperature). Two portions of 5 c c each are removed to 8 x 1 inch pyrex test tubes. The contents are made strongly alkaline with NaOH, and the free ammonia is blown out into flasks, containing exactly 10 c c of N/50 HCl., about 70 c c of water, and a drop of alizarin. The entire amount of nitrogen as ammonia is removed by this air current method in less than 30 minutes. Back titration of the contents of each flask with N/50 NaOH completes the process. Duplicate determinations of duplicate digestion on the same sample may be confidently expected to agree within 0.10 c c N/50 NaOH. Much time is conserved in calculation by the use of tables, which are readily prepared to show at a glance the back titration in terms of N/50 NaOH, and the corresponding number of milligrams of nonprotein nitrogen per 100 c c of culture medium.

Experience indicates that no precautions other than reasonable care in measurements and ordinary skill in Kjeldahl determinations are necessary to insure the precision of results indicated in the foregoing. The titration method

[16] The Folin phosphoric-sulphuric acid digestion mixture has been found to be somewhat less suitable for culture mediums than the Kjeldahl-Gunning digestion mixture. Undoubtedly the nitrogenous constituents of cultural mediums are more difficult to break up than those of the blood for which the Folin mixture is excellent.

offers some advantages over the Nesslerization of the digestion mixture as carried out by Folin. When large numbers of determinations are made (the writer has frequently carried out 50 unaided in a morning), the cost of the Nessler reagent becomes a potent factor.

It is clear that the difference between the "nonprotein nitrogen" and the total nitrogen represents nitrogenous substances of molecular aggregation precipitable by the tungsten. This fraction is advantageously termed "protein nitrogen."

Amino Nitrogen.—Henriques,[17] and Henriques and Sörenson,[18] have taken advantage of the reaction between formaldehyde and free NH_2 groups in proteins and protein derivatives to produce methylated nitrogenous groups, thus destroying the basicity of the NH_2 group, in their method of "formol titration." The reaction is as follows:

$$R.\ CH_2.\ CHNH_2.\ COOH + HCOH = R.\ CH_2.\ CHN : CH_2.\ COOH + H_2O.$$

The basicity of the amino group being eliminated, the full acidity of the carboxyl group becomes titratable, and it may be measured quantitatively by titration with standard caustic soda solution, using phenolphthalein as an indicator. It is clear that free ammonia that may be present in solution also reacts with formaldehyde—in this case, however, to form hexamethylene tetra amine. A correction should be made for the free ammonia in the determination.[19]

It should be remembered that the only free NH_2 groups are thus determinable; the remaining amino groups of a polypeptid are united chemically to the carboxyl groups, forming the well-known "protein or 'peptid' tie." In a dipeptid, only one free amino group is ordinarily present, and, generally speaking, it is very probable that polypeptids possess for the most part only one free amino group and one free carboxyl group.[20] The remaining amino and carboxyl groups are united in order to link together the amino acids which collectively comprise the

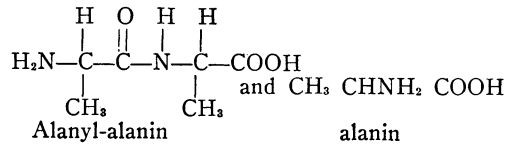
[17] Ztschr. f. physiol. Chem., 1909, 60, p. 1.

[18] Ibid., 1910, 54, p. 120.

[19] It appears probable that much of the ammonia, even in plain gelatin mediums is in combination with CO_2 as ammonium carbonate. The change of the ammonia to hexamethylene tetra-amine by the addition of neutral formaldehyde, therefore, would remove the basicity of the ammonia. Also, the neutralization of basic digestion mixtures of bacterial causation will result in the union of ammonia with the acid (HCl) up to the neutral point of the indicator used. The addition of formaldehyde and the subsequent neutralization of the acidity of the mixture containing free carboxyl groups will probably go to a point where sodium will replace ammonium in ammonium chloride; the slowly liberated ammonia will tend to unite with formaldehyde to form hexamethylene tetra-amine, and in proportion to the ammonia present. If, however, this supposition of the influence of ammonia is incorrect, the net effect on the calculation of the distribution of nitrogen is after all quite small, because the amino nitrogen fraction changes relatively little during the period of incubation.

[20] Of course the dicarboxylic acids, aspartic and glutamic, are specifically exceptions to this NH_2 — COOH ratio.

polypeptid. It is not surprising, therefore, to find that proteins, or even peptones, possess comparatively few free NH_2 groups. Their formol titrations are relatively small. For example, alanin and alanyl-alanin have the same theoretical formol titration. Thus:



have each one free NH_2 group. Hence, their formol titrations would be the same. A determination of the formol titration, and of the "non-protein nitrogen" (correcting both solutions for ammonia), would offer a theoretical means of determining the respective amounts of each in a culture medium. It is very evident that the usual cultural mediums will contain much more "polypeptid" nitrogen than "amino" nitrogen.

Procedure: Ten c c of culture medium are introduced into a beaker, together with about 40 c c of neutral, distilled water. The reaction is brought to the neutral point of phenolphthalein (P_H 8.3) by the cautious addition of N/10 HCl or N/10 NaOH, as may be required. Five c c of formaldehyde, exactly neutral to phenolphthalein, are added to the solution, which immediately becomes acid due to the reduction in basicity through the removal of the NH_2 groups, as outlined in the foregoing.

A second titration to neutrality will measure the acidity of the carboxyl group. As one free COOH occurs for one free NH_2 group in most amino acids and polpeptids,[20] the equivalent amount of standard ammonia corresponding to the amount of standard alkali in the titration after the addition of the neutral formaldehyde solution will be a measure of the "amino nitrogen" of the solution. Duplicate determinations will check with 0.10 c c N/10 NaOH without any difficulty. The precision of this reaction is about that of the nonprotein nitrogen and the total nitrogen determinations, if it is carried out with reasonable care. Rarely solutions are so highly colored that the end point is obscured somewhat.

An alternative procedure, much more time-consuming and presumably somewhat more exact, is the Van Slyke [21] method for the determination of amino nitrogen. The correction to be applied for free ammonia is somewhat more difficult to evaluate, since ammonia reacts relatively slowly with nitrous acid, which is the reagent used in the Van Slyke procedure. Generally speaking, the precision of the Sörensen titration wherever it can be used in the study of the nitrogenous metabolism of bacterial cultures is commensurate with the accuracy attending the parallel development of bacterial growths.

[21] Jour. Biol. Chem., 1912, 12, p. 275.

The amino nitrogen changes in bacterial cultures, as might be expected, are less marked and less significant than the concomitant changes in total nonprotein nitrogen and the gradual accumulation of free ammonia.

Free Ammonia.—Available evidence indicates that the accumulation of free ammonia in cultures of bacteria (probably present in no inconsiderable degree as ammonium carbonate) is very largely, if not practically exclusively, the result of the intracellular utilization of protein or protein derivatives for structural requirements (including the formation of enzymes and other structural replacements) and more particularly for energy. The extrusion of ammonia from the amino acid complex prior to the oxidation of the carbon is known as deamination. It is a measure of the intracellular utilization of amino acids for energy.

The difference in the amount of ammonia formation in protein and protein-carbohydrate mediums, respectively, is an indication of the sparing action of utilizable carbohydrate for protein as a source of energy. The analogy of the sparing action of carbohydrate for protein for bacterial cultures has its analogy in the sparing action of carbohydrate for protein in the human and animal body. Urea in the urine and ammonia in cultures of ordinary bacteria have approximately analogous significance.

Determination of Free Ammonia.—The determination of free ammonia in cultures of bacteria is readily accomplished with the Folin-MacCallum [22] modification of the Folin [23] air current method.

PROCEDURE

Two c c of culture medium are measured accurately with an Oswald pipet into an 8x1 inch pyrex test tube. One c c of an aqueous solution saturated both with sodium carbonate and sodium oxalate is added to the tube, and 1 c c of kerosene [24] to prevent foaming. The tube is closed with a two-hole tightly fitting rubber stopper. Air freed from ammonia by passage through a wash bottle containing 25% H_2SO_4 is led to the bottom of the tube, and bubbled through the solution. The air, containing ammonia, is passed through glass and rubber tubing to a glass tube terminating in a small bulb containing a crown of small openings. This tube reaches to the bottom of a 250 c c Erlenmeyer flask containing exactly 10 c c (or a multiple of 10 c c when necessary) of N/50 HCl, about 65 c c of water (neutral to alizarin), and a drop of aqueous alizarin solution (0.5%). It will be seen that this is a closed system through

[22] Jour. Biol. Chem., 1912, 11, p. 363.

[23] Ztschr. physiol. Chem., 1902, 37, p. 161.

[24] Old kerosene is better than fresh, because some of the more volatile constituents disappear on standing.

which ammonia-free air is forced through an alkaline solution of culture medium containing ammonia. The air current in its passage through the culture medium forces the ammonia (which is in the free state) quantitatively out of the medium, and carries it to the acid in the flask where it is quantitatively absorbed. After 15 minutes, with a fairly brisk air current, the entire amount of ammonia is gathered in the acid flask. The difference in titration between the original amount of N/50 HCl and that remaining free after the absorption of the ammonia as NH_4Cl, gives an accurate measure of the latter. The results of duplicate determinations check readily within 0.1 c c of N/50 alkali on back titration.

A number of details add to the rapidity with which the process may be carried out.

The most satisfactory air pump is the Westinghouse automatic type, driven by steam. A reservoir of 50 to 100 gallons to draw from is a necessity. The air pump will maintain a very nearly uniform pressure in such a tank without any human interference.

The delivery of air is readily accomplished through a long pipe set at 6-inch intervals with two opposite lateral openings. Each opening is controlled with a pet cock, or, better, with a gate valve. From each individual valve the air passes through a 2 liter bottle ⅛ filled with 25% of H_2SO_4. The air is forced through the acid (which frees it from ammonia), and from the bottle it passes to a tube which can be connected with one of the two delivery tubes in the pyrex test tube, as indicated in the foregoing. A battery of 20 pairs of independent air openings, each with its control valve, can be set up on a table less than 14 feet long and 3 feet wide.

For convenience the test tubes containing the solutions to be freed from ammonia can be supported in the double clamp so commonly used for burettes in chemical analyses. The occurrence of the tubes in pairs adds greatly to the convenience of keeping track of duplicate determinations. A pipe ¾ inch in diameter with ½ inch lateral openings, carrying 30 pounds' air pressure, is ample to carry 20 pairs of apparatus at one time. A drain cock should be placed at the distal end of the pipe to permit of the removal of condensation water which usually collects in the system.

The Oswald pipet should deliver exactly 2 c c. Such pipets may be purchased at any reputable chemical supply house. They should be tested for delivery, however. The dispensing of many flasks, each containing exactly 10 c c of N/50 HCl is best accomplished by the use of an automatic 10 c c pipet connected with the reservoir of acid. Such pipets are purchasable, but they must be tested for accurate delivery. Once a perfect pipet is obtained, it saves many times its cost in economy of time.

The N/50 alkali must be prepared, using alizarin as an indicator. Solutions that are the equivalent of N/1 HCl and N/1 NaOH, using phenolphthalein as an indicator, will not be exactly equivalent when they are diluted to N/50 strength. About 39.38 c c of N/1 NaOH will make exactly 2 liters of N/50 NaOH that will agree with N/50 HCl diluted in the proportion of 40 c c N/1 HCl to 1,960 c c of water.

The distilled water must be neutral to alizarin. Not all stills will deliver water of this reaction.

When the ammonia is removed from the culture solution, and the air is turned off, the delivery tube in the flask containing acid must be washed down with distilled water before the solution is titrated, otherwise some acid adheres to the tube and is lost.

Ammonia determinations performed in this manner check with the greatest ease to 0.10 c c of N/50 solution. This degree of precision should be adhered to in the methods described for total nitrogen, nonprotein nitrogen, and ammonia. All of these determinations, it will be remembered, are made with the air current method for removing the ammonia from solution and combining it with N/50 HCl. One c c of N/50 standard alkali corresponds to 0.00028 gm. ammonia nitrogen. Therefore, duplicate determinations check within 0.000,028 gm. nitrogen as ammonia, plus or minus. This is a relatively small error.

SUMMARY

The determination of total nitrogen, "nonprotein nitrogen," amino nitrogen and ammonia nitrogen, as outlined, permits a fractioning or division of the nitrogen in cultural mediums as follows:

1. Total Nitrogen.
2. Protein Nitrogen: Obtained as the difference between total nitrogen and nonprotein nitrogen.
3. Nonprotein Nitrogen.
4. "Polypeptid Nitrogen:" Obtained as the difference between the nonprotein nitrogen and the sum of the amino and ammonia nitrogen.
5. Amino Nitrogen: Obtained from the formol titration after subtraction of the free ammonia.
6. Free Ammonia: Obtained by the air current method as outlined. The relations of the several fractions are indicated briefly in the following examples:

TABLE 3

RELATIONS OF THE SEVERAL FRACTIONS

	A	B	C
Total nitrogen	1.064	1.064	1.064
Protein nitrogen	0.762	0.863[25]	0.885[25]
Nonprotein nitrogen	0.302	0.201	0.179
"Polypeptid nitrogen"	0.213	0.092	0.092
Amino nitrogen	0.032	0.030	0.037
Ammonia nitrogen	0.057	0.079	0.050
Reaction	+0.60	−1.70	+5.80
P_H	7.0	8.1	4.9

A, control, uninoculated medium.
B, plain, sugar-free nutrient broth.
C, plain, sugar-free nutrient broth: (A), containing in addition 1% glucose; (B and C), parallel 7-day cultures of Bacillus coli.

The changes are readily explainable and need no comment. The least significant change appears to be that induced in the amino nitrogen fraction.

[25] Increase due chiefly to protein in the bacterial cells.

More energetic proteolytic bacteria, such as B. proteus, induce far greater changes, thus:

TABLE 4

CHANGES PRODUCED BY B. PROTEUS

	Control	A, Plain Gelatin	B, 1.5% Glucose Gelatin
Total nitrogen	1.001	1.001	1.001
Protein nitrogen	0.721	0.038	0.732
Nonprotein nitrogen	0.280	0.963	0.269
Polypeptid nitrogen	0.209	0.696	0.186
Amino nitrogen	0.039	0.036	0.044
Ammonia nitrogen	0.032	0.204	0.039

7-day incubation.

Here again the amino nitrogen fraction changes but little, although the free ammonia in the plain gelatin (deamination) increases very much indeed. In glucose gelatin the deamination is minimal.

That the increase in free ammonia is due to the intracellular utilization of the protein for energy is shown by the following experiments, in which 5 c c of culture A and of culture B, respectively, were placed in 95 c c of 10% carbol gelatin. No bacterial development took place of course. Incubation was practiced for 3 days to allow the enzyme in "A" to act.

TABLE 5

SEVEN-DAY CULTURE IN CARBOL GELATIN

	Control	A	B
Total nitrogen	1.330	1.330	1.330
Protein nitrogen	1.061	0.332	1.051
Nonprotein nitrogen	0.269	1.008	0.279
Polypeptid nitrogen	0.211	0.916	0.222
Amino nitrogen	0.040	0.073	0.040
Ammonia nitrogen	0.018	0.019	0.017

Here, as might be confidently expected, the deamination failed to take place. This shows strikingly what the sparing action of utilizable carbohydrate for protein really means in the nitrogen spectrum of a bacterial culture. The action of the enzyme in "A" is very clearly indicated.